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14. ABSTRACT Lowering androgen levels by orchiectomy (ORX) or reversible pharmacological treatment is a key therapeutic goal in prostate cancer patients. This life prolonging treatment is accompanied by the adverse side effects of increased adiposity, loss of muscle mass, and osteoporosis that negatively affect quality of life. Thus, there is a need for therapeutic agents that improve the quality of life for prostate cancer patients treated with androgen deprivation therapy (ADT). We hypothesize that mice undergoing ORX have reduced muscle specific force due to calcium leak through RyR1, which is caused by high levels of TGFβ released from the bone during ADT-related bone loss. We studied the effect of ORX in young and aged mice over a period of 20 weeks after surgery. We found that ORX mice had decreased muscle weight, mid-calf cross-sectional area, forelimb grip strength, and bone mineral density (BMD), and increased total body fat content. However, muscle specific force was not decreased in ORX mice compared to sham operated mice. In future experiments adult mice will be treated with ORX and/or androgen receptor antagonist, to better mirror the clinical approach in prostate cancer patients. Because in our experiments the lowest level of BMD was reached at 4 weeks after ORX, we will measure muscle specific force at 4 weeks and 8 weeks after surgery to determine if an early calcium leak could be causing long-term effects, such as decreased muscle mass, body weight and forelimb grip strength.					
15. SUBJECT TERMS Prostate Cancer, Androgen, Orchiectomy, Androgen Deprivation Therapy, Ryanodine Receptor 1, RyR1, Calstabin 1, Muscle Dysfunction, S107, Calcium, Ca2+					
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1. INTRODUCTION

Androgen deficiency occurs as a consequence of hypogonadism, certain cancer treatments, or normal aging in men. Lowering androgen levels, by orchiectomy (ORX) or reversible pharmacological treatment, is a key therapeutic goal of androgen deprivation therapies (ADT) used in patients with androgen-dependent cancers, such as prostate cancer. Unfortunately, this life prolonging treatment is accompanied by the adverse side effects of androgen deprivation resulting in increased adiposity, loss of muscle mass, and osteoporosis that negatively impact quality of life. Thus, there is a need for non-androgenic therapeutic agents that reduce the severity of such effects and improve the quality of life for prostate cancer patients treated with ADT. Double-blinded studies have unequivocally demonstrated that, even in hypogonadism or normal aging derived androgen deficiency, androgens regulate muscle mass in humans (1). Although, the effect of androgens on subsequent muscle strength and function is less clear. Some studies have demonstrated enhanced muscle strength following androgen administration (2-6). However, other studies have failed to detect a significant functional effect of androgen therapy despite gain in muscle mass (7-10). The reasons for this discordance are unclear and it is, therefore, crucial to understand changes occurring at the cellular level in muscle tissue in the setting of androgen deprivation.

One of the effects of androgen deficiency at the cellular level is the altered expression of molecules involved in the cellular redox system. Mancini et al. reported that CoQ10 and total antioxidant capacity were lower, although not significantly, in hypogonadal compared with eugonadal patients (11). In a study of gene expression in androgen deficiency, Pang et al. found that several antioxidant genes were modulated after castration in rat ventral prostate (12). Among them, thioredoxin, peroxiredoxin 5, glutathione peroxidase 1, superoxide dismutase 2, and 15kDa selenoprotein are genes functionally related to cellular redox regulation that were down-regulated after castration. In contrast, other redox-related genes such as glutathione reductase, microsomal glutathione-S transferase, and epoxide hydrolase were up-regulated after castration. Alteration of antioxidant proteins can cause oxidative stress to cells because the balance of reactive oxygen species and antioxidant system is lost.

Maladaptive cAMP-dependent protein kinase A (PKA)-mediated phosphorylation and redox-dependent modifications (cysteine nitrosylation and oxidation) of the Ryanodine receptor 1 (RyR1) have been linked to a pathologic intracellular sarcoplasmic reticulum (SR) calcium leak and contractile dysfunction in chronic muscle fatigue, heart failure and muscular dystrophy (13-16). RyR1 is the skeletal muscle sarcoplasmic reticulum calcium release channel required for muscle contraction. RyR1 is a homotetrameric macromolecular protein complex that includes four RyR1 monomers (565kDa each), the RyR1 channel-stabilizing subunit calstabin1 (FKBP12), kinases, a phosphatase (PP1), a phosphodiesterase (PDE4D3), and calmodulin13. Both nitrosylation and oxidation have been shown to affect skeletal muscle RyR1 function and Ca^{2+} signaling (17-19). S-nitrosylation of RyR1 has been shown to disrupt the interaction between RyR1 and calstabin1, resulting in channels that can leak Ca^{2+} into the cytoplasm, activation of the Ca^{2+} -dependent protease calpain that causes muscle damage, and impaired muscle function (13,15). Kovacheva et al. had previously demonstrated that aging resulted in an increase of 4-hydroxynonenal protein adducts, a biomarker of oxidative stress, and that testosterone treatment reduced this age-associated increase in oxidative stress (20). Recently, it has been shown that RyR1 oxidation causes intracellular Ca^{2+} leak and muscle weakness in aging (21). Aged mice have a significantly lower serum concentration of testosterone than young mice (22), but it remains to be elucidated whether the decline in androgen levels plays a role in the oxidation of RyR1. Rycal S107 is a novel therapeutic agent that has been shown to stabilize binding of calstabin1 to RyR1, reduce intracellular Ca^{2+} leak, decrease reactive oxygen species (ROS), and enhance tetanic Ca^{2+} release, muscle-specific force, and exercise capacity in aged mice (23). If muscle dysfunction in androgen deficiency is mediated through oxidation of RyR1, S107 may be an effective therapy, especially when the patient cannot tolerate androgen replacement or when this is contraindicated, as in the setting of prostate cancer.

Our previous data shows that muscle function is reduced in female mice with breast cancer metastases to bone. This muscle dysfunction was associated with oxidation and nitrosylation of RyR1, depletion of calstabin1, and dysmorphic and hypertrophied mitochondria. Preliminary data in mice with prostate cancer bone metastases

indicate that muscle function is reduced in male mice that underwent ORX, compared with Sham mice, and that muscle function improves in castrated mice that are treated with either an inhibitor of osteoclastic bone resorption (zoledronic acid) or an inhibitor of transforming growth factor beta (TGF- β) receptor I kinase (SD208). Since it is well established that oxidative stress is elevated in cancer cachexia, we hypothesized that a similar mechanism could underlie impaired skeletal muscle function associated with androgen deprivation. Further research is needed to clearly identify the effects on muscle function specific to androgen deprivation. Hence, our study aims to characterize ADT effect on muscle function and its mechanism in non-tumor bearing mice.

2. KEYWORDS

Prostate Cancer, Androgen, Orchiectomy, Androgen Deprivation Therapy, Ryanodine Receptor 1, RyR1, Calstabin 1, Muscle Dysfunction, Rycal, S107, Calcium, Ca²⁺.

3. OVERALL PROJECT SUMMARY

Task 1: Training in cellular techniques.

Training at Columbia University in measuring RyR1 oxidation, nitrosilation, and PKA phosphorylation in skeletal muscles. Quantification of depletion of the channel stabilizing subunit calstabin1 (FKBP12) from skeletal RyR1 by immunoprecipitation techniques, assessing channel open probability at low activating cis [Ca²⁺] = 150nM to determine whether skeletal muscle RyR1 from castrate or intact mice are “leaky”. Measuring mitochondrial superoxide production by using the cell permeable fluorescent indicator MitoSOX Red.

*Completion of this task will give expertise in cellular & biochemical techniques, which is crucial to the integrity of the study. *Collaborator: Dr. Andrew Marks.*

1. Training in cellular techniques was performed in the Guise Laboratory under the mentorship of Dr. David Waning, a member of the Guise Laboratory. Dr. Waning was trained in the Marks Laboratory, at Columbia University, as a representative of the Guise Laboratory, in techniques used to assess RyR1 functionality. Dr. Waning trained Dr. Antonella Chiechi (P.I. of the present project) in RyR1/Calstabin1 co-immunoprecipitation techniques, RyR1 oxidation and nitrosilation detection techniques, Ca²⁺ leak from SR vesicles measurement techniques and oxidative stress level measurement techniques. For training purposes, snap-frozen mouse muscles (tibialis anterior) from previous studies were used. Muscles were grinded and lysed in specific protein extraction buffers (recipes developed by the Marks Laboratory specifically for RyR1/calstabin1 immunoprecipitation). Immunoprecipitation of RyR1 followed by western blot for RyR1 and calstabin1 detection was performed to determine levels of calstabin1 binding RyR1 in muscle lysates from control mice and tumor bearing mice. To measure RyR1 nitrosilation we performed RyR1 immunoprecipitation in muscle lysates followed by western blot. The blots were then probed with CysNO to detect nitrosilated cysteine residues. To measure RyR1 oxidation, muscle lysates were immunoprecipitated with anti-RyR1 antibody and incubated with a DNPH solution to derivatize carbonyl groups, which result from oxidative modification of proteins. Treated lysates were used to perform western blot and the blots were then probed with anti-DNP antibody to detect carbonyl groups. Detection of PKA phosphorylation of RyR1 was not performed during the training because not strictly necessary to the project. RyR1 oxidation and nitrosilation and loss of calstabin1 binding constitute the accepted biochemical signature for “leaky” RyR1.

Assessment of RyR1 channel open probability at low activating $\text{cis [Ca}^{2+}]$ (150nM) requires specific instrumentation that is not available at Indiana University. In alternative, Dr. Chiechi learned how to perform measurement of Ca^{2+} leak from SR vesicles using a dynamic fluorescence assay, an assay also used in the Marks Laboratory to assess RyR1 functionality. For this assay, murine skeletal muscles were processed to obtain microsomes containing, among other proteins, RyR1 and SERCA. A fluorescent solution was added to the microsomes and CaCl_2 was successively added to it. Fluorescent emission was measured with a fluorimeter until stable: emission decreases as SERCA pumps Ca^{2+} into the microsomes. Thapsigargin was then added to block SERCA and fluorescent emission was measured until stable: emission increases as “leaky” RyR1 releases Ca^{2+} from the microsomes into the solution. MitoSOX is a fluorescence based assay used to detect mitochondrial ROS production in live cells, therefore not suitable for use in our frozen muscle samples. To overcome this issue, in the training, we used OxiSelect Protein Carbonyl ELISA Kit to measure oxidative stress levels. For training purposes, frozen murine muscle samples, deriving from previous in vivo experiments, were lysed and protein lysates were used for the assay. In this assay, protein carbonyls present in protein lysates (due to oxidative modifications) are derivatized to DNP via incubation with DNPH and an anti-DNP antibody is used to detect the derivatized carbonyls in the sample. Protein carbonyl groups are a direct measure of oxidative stress in the samples.

Task 2: Training in animal studies.

a. Training in performing the following: mouse ORX, dissection of fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles, quantifying the muscle specific force (the absolute force normalized to the muscle size), measuring body composition and bone densitometry using a mouse DXA scan (PIXImus II, GE Lunar), assessing muscle cross sectional area in live mice using a microCT scanner, analyzing skeletal muscle mitochondria via electron microscopy (including quantification).

b. Analyzing adequacy of testosterone replacement by subcutaneous insertion of a small slow release pellet containing testosterone releasing 25 $\mu\text{g/day}$ during 60 days, corresponding to 2mg/kg/day in ORX mice, followed by obtaining blood samples by cardiac puncture immediately after death and measuring serum testosterone levels at 15, 30 and 60 day point by RIA; measuring plasma S107 levels to ensure adequate levels are obtained by mixing S107 (50mg/kg/day) in the drinking water.

Animal Usage: 7 C57BL/6 mice

*Completion of this task will give expertise in animal studies, which is crucial to the integrity of the study. *Collaborator: Dr. Khalid Mohammad.*

2a. Dr. Chiechi was trained by Dr. Mohammad to perform orchiectomy (ORX) in mice using aseptic technique. Mice are anesthetized and shaved over the ventral side of the scrotum to expose skin. Shaved skin is swabbed with 70% (v/v) ethanol followed by sterile PBS. The anaesthetized mouse is placed on the operating table on its back. A single medial incision is made on the ventral side of the scrotum (0.5 cm) penetrating the skin. The testicular fat pad on one side is pulled through the incision using forceps and the testicular content is exposed. Cauda epididymis and caput epididymis are severed and testis is removed gently with small scissors. The excision area is cauterized with mini-surgical cautery. The remaining content of testicular sac is placed back. The procedure is repeated for the other testicle through the same medial incision. The skin is closed with non-absorbable sutures. Dr. Waning trained Dr. Chiechi in dissecting fast-twitch extensor digitorum longus (EDL) muscle from the mouse hind limb and performing the ex-vivo contractility assay to measure muscle specific force. The soleus muscle will not be used in this study: in previous studies we have determined that fast-twitch muscles are affected the most by muscle weakness and dysfunction and RyR1 is expressed in a much larger amount in these muscles compared to slow-twitch muscles. The hind limb is removed from the mouse body and the skin is removed from the limb. The hind limb is secured with needles on a wax

block in a tray filled with Tyrode's solution (to preserve and equilibrate the muscles for the following muscle physiology experiments). Under a microscope, the tibialis anterior (TA) muscle is cut with micro dissection scissors at the distal insertion and lifted to allow access to the EDL muscle. Using nylon suture, two small stainless steel hooks are tied to the tendons, one at the proximal insertion of the EDL muscle and one at the distal insertion, making sure that the suture is on the tendon and not touching the muscle. The EDL muscle is then removed with micro dissection scissors and ready for immediate use in the contractility assay. The EDL muscle is mounted on a muscle physiology testing system (Aurora Scientific), between a force transducer and an adjustable hook. Electric stimulation of the intact muscle leads to contraction and the force generated is recorded via data acquisition hardware and software for further analyses. To quantify the specific force, the absolute force is normalized to the muscle size, calculated as the muscle weight divided by the muscle length and multiplied by a muscle density constant of 1.056kg/m.

Dr. Chiechi was trained by Dr. Mohammad in using a mouse DXA scan (PIXImus II, GE Lunar) and in analyzing the resulting data and images using the PIXImus II associated software in order to measure body composition (lean and fat content) and bone mineral density. Dr. Mohammad also trained Dr. Chiechi in using a MicroCT scanner (Scanco VivaCT40) for in vivo imaging of mice and in analyzing the resulting images to determine the mid-calf muscle cross-sectional area using the Scanco VivaCT40 associated software.

Dr. Chiechi was not trained in analyzing skeletal muscle mitochondria via electron microscopy since the technique will not be utilized in this study because very expensive and not necessary to determine muscle dysfunction in this model as determined from previous studies.

- 2b.** Task 2b will not be completed. Testosterone administration is not a suitable therapy for prostate cancer patients and, therefore, has no clinical relevance. This study aims to investigate the mechanism of ADT-related muscle dysfunction and identify new approaches to ameliorate muscle function and quality of life in patients with prostate cancer undergoing ADT. For these reasons, we decided not to perform the testosterone replacement experiment and, instead, focus on clinically applicable treatments. In fact, we will test the effects of zoledronic acid, a conventionally used bisphosphonate to treat bone loss in ADT treated patients, on muscle function when administered alone or in combination with S107. S107 will not be administered to the mice in the drinking water as described in the original project, but via an osmotic mini-pump (Alzet) to be inserted sub-cutaneous in the mice, allowing for a more reliable dose administration and reducing variability. The osmotic mini-pump has 100µl capacity with duration of release from 1-4 weeks. This will insure a constant release of S107 at 50mg/kg/day for 28 days. Dr. Chiechi will be trained in the surgical insertion of osmotic mini-pumps by Dr. Mohammad.

Task 3: Determine whether androgen deprivation causes reduced muscle specific force (muscle force normalized to the muscle size) in young and old mice & whether RyR1 dysfunction is an underlying mechanism responsible for the decrease in muscle function in androgen deprivation.

In phase 1, mice will be randomly assigned to either sham-operated (SHAM) or castrated (ORX) experimental groups in the young and aged category. Specific measurements of body composition, muscle mass, muscle function and biochemical analysis of RyR1 will be made over the next 12 weeks. At the end of the 12 weeks, mice will be euthanized, and muscle harvested and subjected to functional and biochemical assessment (described in task 1 & 2).

Animal Usage: 60 C57BL/6 mice

*Completion of this task will demonstrate muscle dysfunction occurring as a consequence of androgen deprivation and identify RyR1 dysfunction as the underlying cause. *Collaborator: Dr. Andrew Marks.*

- 3.** Thirty 9-week-old male C57BL/6 mice (young mice) and 30 22-week-old male C57BL/6 mice (aged mice) were used for task 3. At baseline, weight and grip strength were measured for all mice, mice were imaged by DXA and MicroCT and underwent periorbital blood withdrawal. Fifteen young mice and 15 aged mice underwent orchiectomy, while 15 young and 15 aged mice underwent sham surgery. Body

weight was measured weekly and grip strength was measured biweekly for the entire length of the experiment. DXA imaging was performed at week 4, 8 and 20 in young mice and at week 4 and 16 in old mice. MicroCT imaging was performed at week 8 and 20 in young mice and at week 16 in old mice. Young mice were euthanized 20 weeks after ORX/sham surgery, while old mice were euthanized 16 weeks after ORX/sham surgery due to profound weight loss. At euthanasia, blood was collected from all mice by pericardiac puncture, tibialis anterior (TA), extensor digitorum longus (EDL), soleus and gastrocnemius were harvested and weighed. The ex-vivo contractility assay was performed on the EDL muscle at euthanasia.

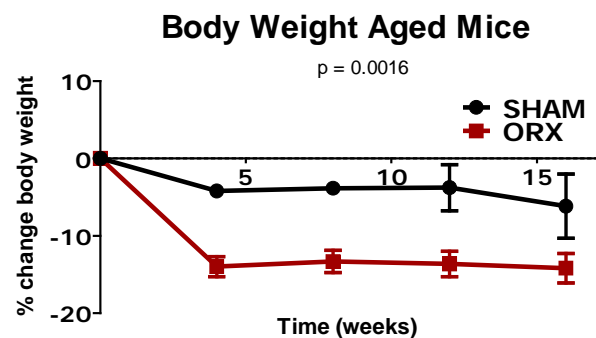
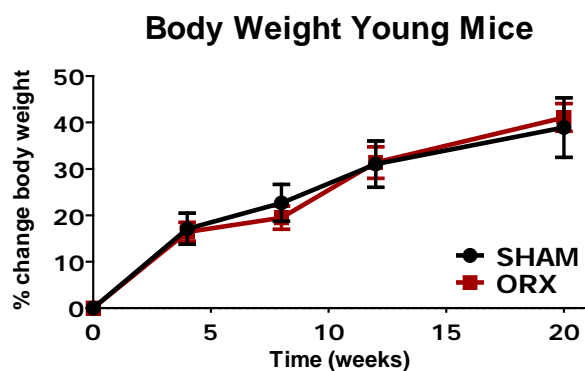
Task 4: Analyzing the phase 1 data.

Analysis of data from phase 1 in consultation with the IUSCC Biostatistics Core. Phase 2 will be started only if there is a statistically significant difference in muscle function between hypogonadal and eugonadal mice in the phase 1 experiment.

*Completion of this task will provide justification for the phase 2 experiment.

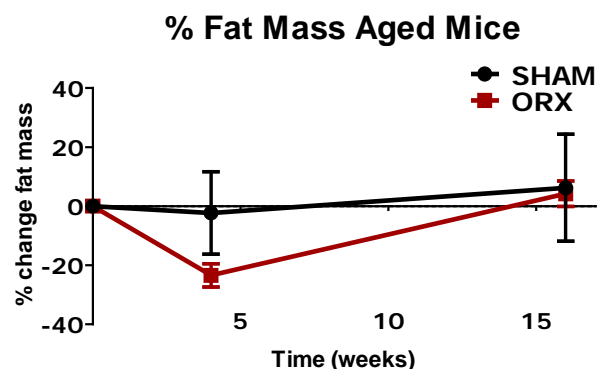
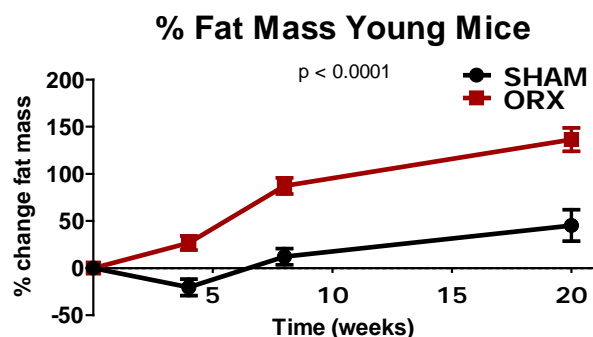
4. Body weight

All young mice gained body weight during the course of the experiment. Young ORX mice showed no statistically significant difference in % change body weight when compared to young sham mice ($p=0.9456$, 2way ANOVA). All aged mice lost body weight during the course of the experiment, with aged ORX mice losing more body weight than aged sham mice ($p=0.0016$, 2way ANOVA).



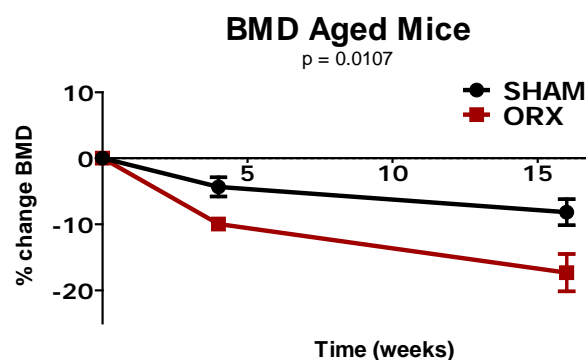
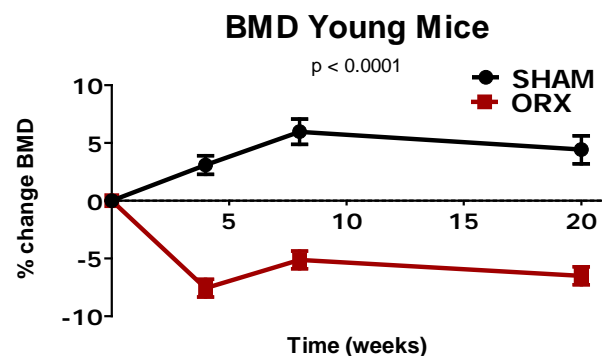
Fat content

Percent fat content (expressed as % change fat mass), measured by DXA, was significantly increased in young ORX mice compared to young sham mice ($p<0.0001$, 2way ANOVA). In aged mice, no difference in % fat content was measured between ORX and sham operated mice ($p=0.4927$, 2way ANOVA).



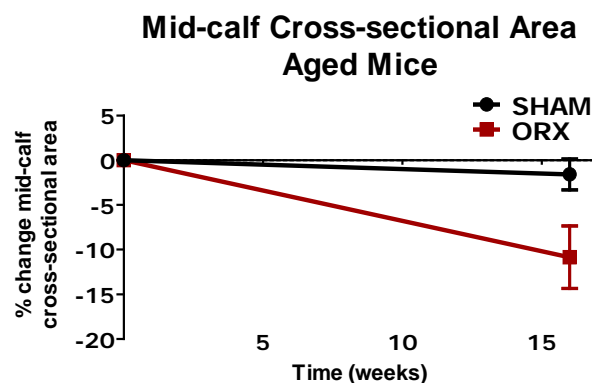
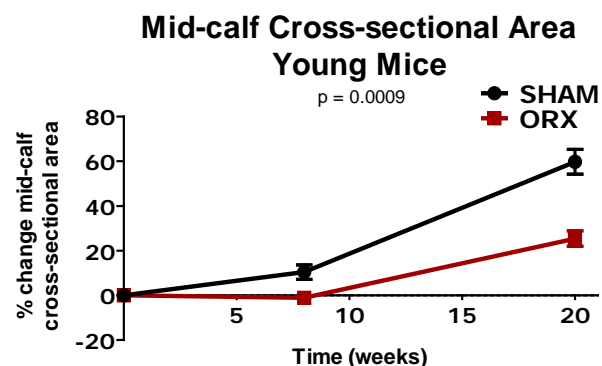
Bone mineral density

Bone mineral density (BMD), measured by microCT, was decreased in ORX mice compared to sham operated mice in both young and aged mice (respectively, $p < 0.0001$ and $p = 0.0107$, 2way ANOVA).



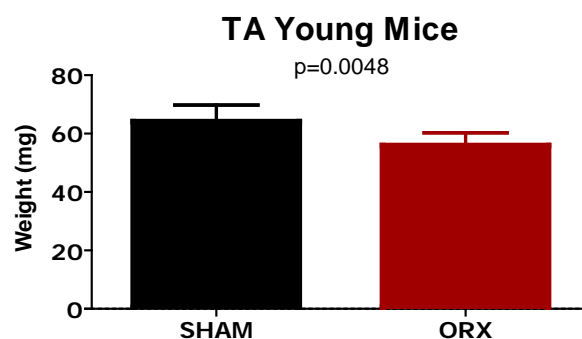
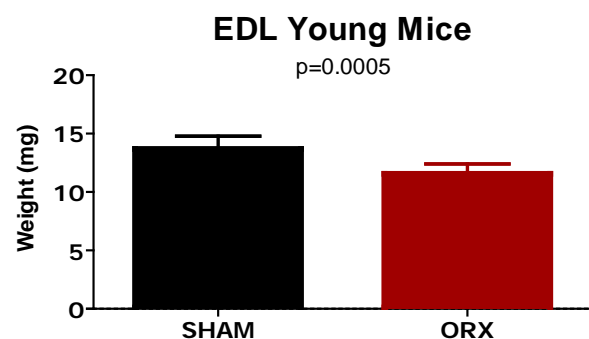
Mid-calf cross-sectional area

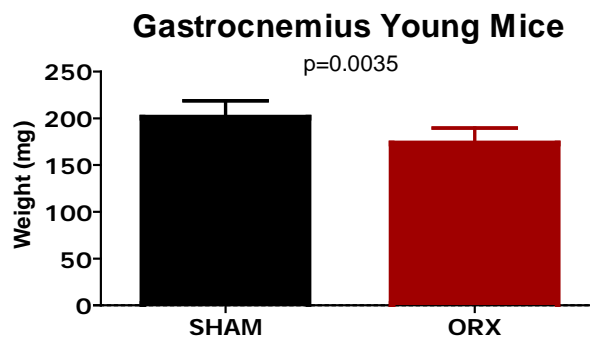
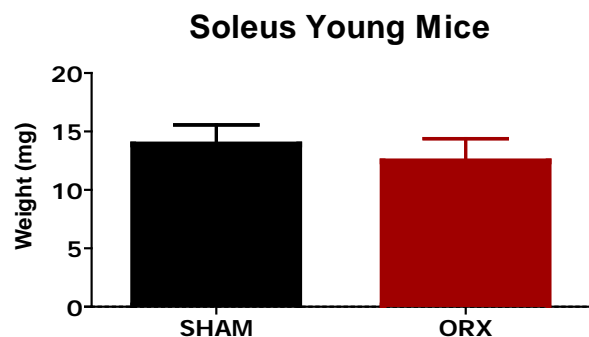
Mid-calf cross-sectional area, measured by microCT, was decreased in young ORX mice compared to young sham mice ($p = 0.0009$, 2way ANOVA). Aged ORX mice showed a decreasing trend for mid-calf cross-sectional area when compared to aged sham operated mice, although the difference is not significant at the statistical analysis ($p = 0.1205$, 2way ANOVA).



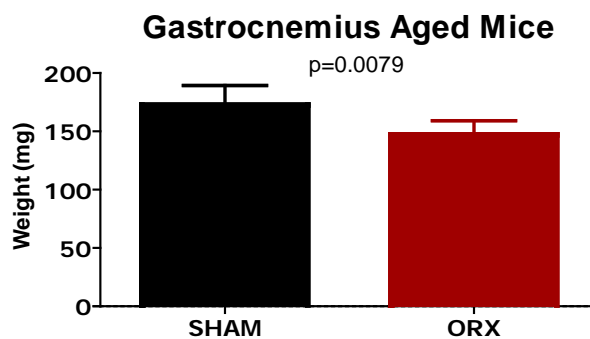
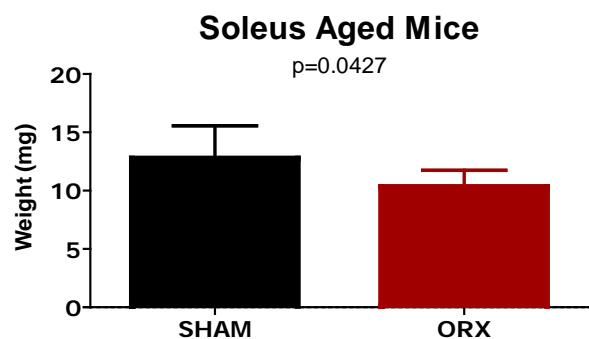
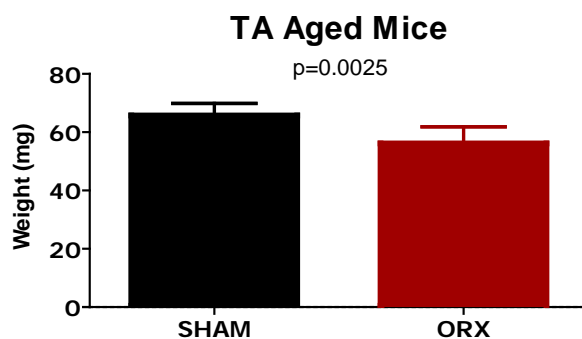
Muscle weight

EDL, TA and gastrocnemius weight was decreased in young ORX mice compared to young sham mice (respectively $p = 0.0005$, $p = 0.0048$, $p = 0.0035$, Mann-Whitney). Soleus weight was not statistically different between ORX and sham mice in the young group ($p = 0.0571$, Mann-Whitney).



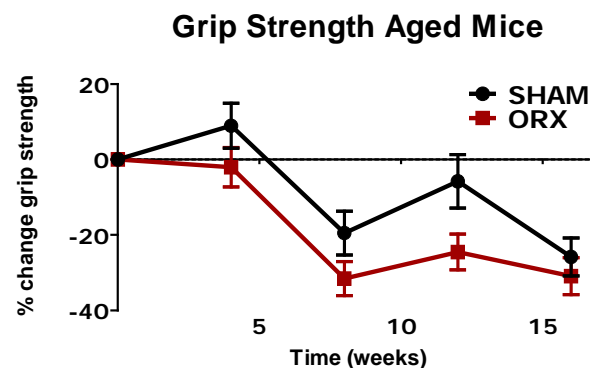
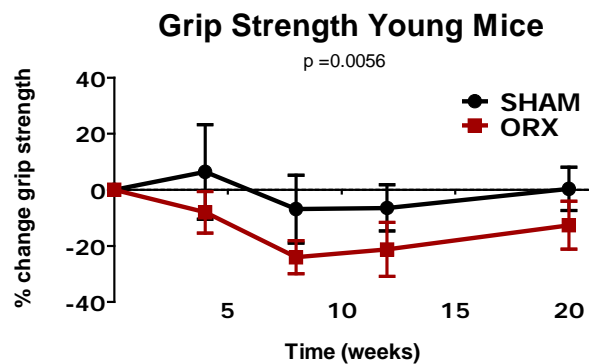


EDL, TA, soleus and gastrocnemius weight was decreased in aged ORX mice compared to aged sham operated mice (respectively p=0.0151, p=0.0025, p=0.0427, p=0.0079, Mann-Whitney).



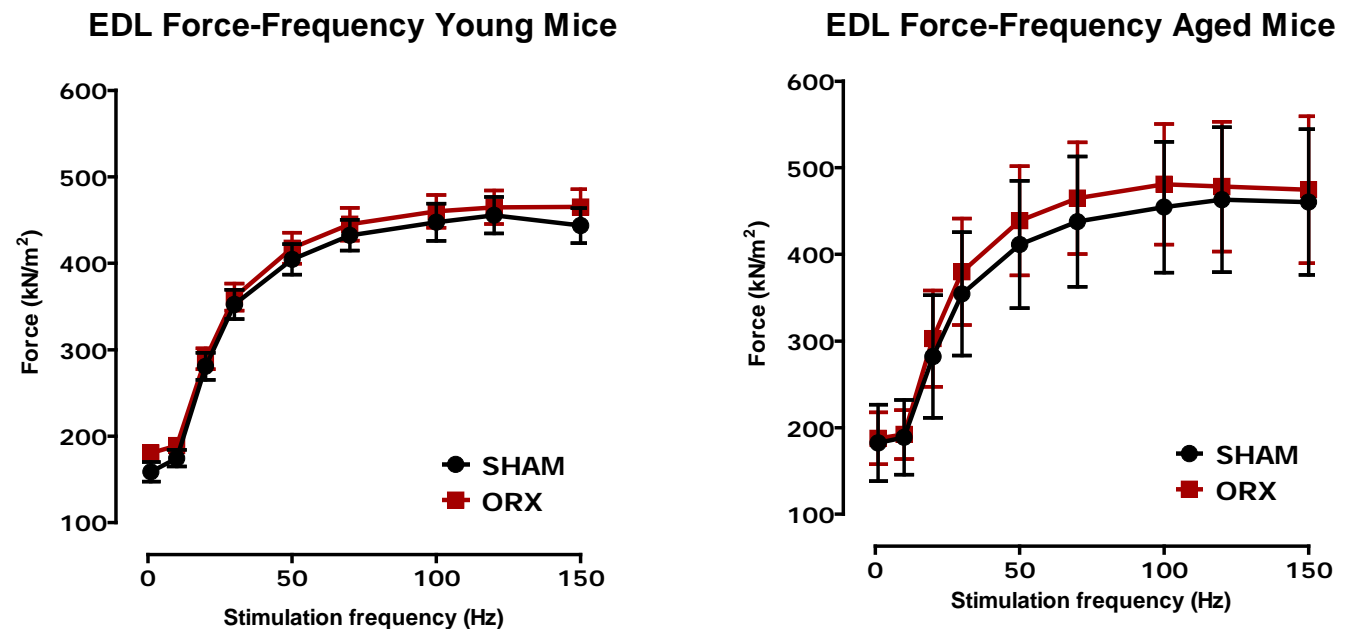
Grip strength

Forelimb grip strength was decreased in young ORX mice compared to young sham mice (p=0.0056, 2way ANOVA). All aged mice showed a decrease in forelimb grip strength during the course of the experiment, although there was no statistically significant difference between aged ORX mice and aged sham mice (p=0.078, 2way ANOVA).



Muscle specific force

In young mice, there was no difference in EDL muscle specific force between ORX and sham operated mice ($p=0.0858$, 2way ANOVA). Aged mice showed high intra group variability and no difference between ORX and sham mice in EDL muscle specific force ($p=0.1246$, 2way ANOVA).



Problems/Changes

Task 1

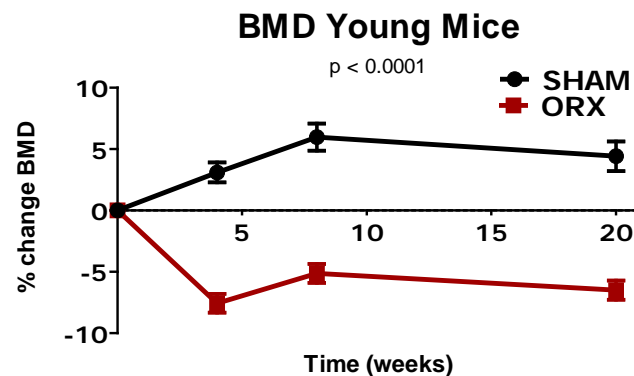
Training in cellular techniques was not performed by Dr. Chiechi in the Marks Laboratory, at Columbia University, as stated in Task 1. The training was, instead, performed in the Guise Laboratory under the mentorship of Dr. David Waning, a member of the Guise Laboratory. Dr. Waning was trained in the Marks Laboratory, at Columbia University, as a representative of the Guise Laboratory, in techniques used to assess RyR1 functionality. Dr. Waning trained Dr. Antonella Chiechi (P.I. of the present project) in RyR1/Calstabin1 co-immunoprecipitation techniques, RyR1 oxidation and nitrosylation detection techniques, Ca^{2+} leak from SR vesicles measurement techniques and oxidative stress level measurement techniques.

Task 2b

Task 2b will not be completed. Testosterone administration is not a suitable therapy for prostate cancer patients and, therefore, has no clinical relevance. This study aims to investigate the mechanism of ADT-related muscle dysfunction and identify new approaches to ameliorate muscle function and quality of life in patients with prostate cancer undergoing ADT. For these reasons, we decided not to perform the testosterone replacement experiment and, instead, focus on clinically applicable treatments. In fact, we will test the effects of zoledronic acid, a conventionally used bisphosphonate to treat bone loss in ADT treated patients, on muscle function when administered alone or in combination with S107. S107 will not be administered to the mice in the drinking water as described in the original project, but via an osmotic mini-pump (Alzet) to be inserted sub-cute in the mice, allowing for a more reliable dose administration and reducing variability. The osmotic mini-pump has 100 μ l capacity with duration of release up to 4 weeks. This will insure a constant release of S107 at 50mg/kg/day for 28 days. Dr. Chiechi will be trained in the surgical insertion of osmotic mini-pumps by Dr. Mohammad.

Overall project

Despite the animal experiments evidenced reduction in muscle mass, forelimb grip strength, and bone mineral density, ORX mice did not show a decrease in EDL specific force. Based on our previous experience with estrogen deprived mice and in line with clinical ADT approach in patients, we will repeat the experiment pairing ORX with an androgen receptor (AR) antagonist (flutamide) to obtain complete androgen deprivation. We will use adult mice (17 weeks-old) with completely developed gonads and stable androgen levels, which is the most similar condition to the one of prostate cancer patients who undergo ADT. We will use nude athymic mice because we have more historical muscle specific force data in this host and because it is a suitable host to be used for a prostate cancer model to further the study in the future. We will euthanize the mice at 2 different time points: 4 weeks and 8 weeks after surgery, based on results from the completed experiment. Bone loss due to ADT generate high levels of TGF β , which, in previous experiments, has been proved to cause reduction in muscle specific force in mice (Waning DL et al. Nature Medicine, in press). Bone mineral density data from the completed experiment show that, in young mice, the lowest BMD is reached 4 weeks after ORX and that BMD values remain stable in the following weeks.



Our hypothesis is that high levels of TGF β , due to bone loss, cause oxidation of RyR1, calcium leak, and reduction in muscle specific force. This reduction in muscle specific force is transient because related to TGF β levels, but it may cause long-term reduction in muscle mass, due to reduced muscle activity, and consequent long-term reduction of body weight and forelimb grip strength. We also hypothesize that treatment with S107 and/or Zoledronic acid will increase (or prevent reduction of) muscle specific force in mice undergoing ADT, and reduce the long-term effects on muscle mass and forelimb grip strength. Therefore, we propose new experiments as described below.

Experiment 1

To test the effect of ADT (by ORX alone or ORX + Flutamide) on muscle specific force in male athymic mice $n=20/\text{group} \times 4 \text{ groups} = 80 \text{ total}$

- Nine week old mice will be ordered.
- After arrival, mice will be acclimated for 2 days.
- Mice will be aged for 8 weeks, until adult (i.e. 17 weeks old).
- 49 days after arrival blood sample will be collected and mice will be weighed.
- 52 days after arrival baseline μCT
- 53 days after arrival DXA and grip strength will be performed for all mice.
- 56 days after arrival orchiectomy (ORX)/Sham surgery and insertion of mini-pumps will be performed consecutively on all mice and drug treatments will commence for all groups.
- All treatment will start 56 days after arrival

Group A: Sham surgery + vehicle (PBS) by s.c. injection.

Group B: ORX + vehicle (PBS) by s.c. injection.

Group C: Sham surgery + Flutamide (45mg/kg/day) by s.c. injection.

Group D: ORX + Flutamide (45mg/kg/day) by s.c. injection.

- Mice will be weighed 2-3 times/week
- All mice will be tested for grip strength weekly
- Mice will be scanned by DXA every 2weeks and by microCT every 4 weeks for the length of the experiment.
- 10 & 3 days before euthanasia all mice will be injected with calcein IP.
- Mice will be euthanized at 4 weeks (10 mice per group) and 8 weeks (10 mice per group) after surgery.
- All bones and organs will be harvested for histological and histomorphometrical analysis.
- Right EDL muscle will be used for ex vivo contractility assay.
- Left EDL muscle will be used for RyR1/Calstabin1 biochemistry.

If Experiment 1 demonstrates reduction in muscle specific force in mice undergoing ORX and/or ORX + Flutamide, then Experiment 2 and Experiment 3 will be performed as described below.

Experiment 2

To test the effect of S107 and Zoledronic Acid (ZA), alone or in combination, on muscle weakness caused by ADT (by ORX alone or ORX + Flutamide) in male athymic mice n=20/group x 8 groups = 160 total

- Nine week old mice will be ordered.
- After arrival, mice will be acclimated for 2 days.
- Mice will be aged for 8 weeks, until adult (i.e. 17 weeks old).
- 49 days after arrival blood sample will be collected and mice will be weighed.
- 52 days after arrival baseline μ CT.
- 53 days after arrival DXA and grip strength will be performed for all mice.
- 56 days after arrival orchiectomy (ORX)/Sham surgery and insertion of mini-pumps will be performed consecutively on all mice and drug treatments will commence for all groups.
- All treatment will start 56 days after arrival

Group A: ORX + vehicle (PBS) by s.c. injection.

Group B: ORX + Flutamide (45mg/kg/day) by s.c. injection + vehicle (PBS) by s.c. injection.

Group C: ORX + ZA (5 μ g/kg/ 3 times/week) by s.c. injection + vehicle (PBS) by s.c. injection.

Group D: ORX + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group E: ORX + Flutamide (45mg/kg/day) by s.c. injection + ZA (5 μ g/kg/ 3 times/week) by s.c. injection + vehicle (PBS) by s.c. injection.

Group F: ORX + Flutamide (45mg/kg/day) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group G: ORX + ZA (5 μ g/kg/ 3 times/week) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group H: ORX + Flutamide (45mg/kg/day) by s.c. injection + ZA (5 μ g/kg/ 3 times/week) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

- Mice will be weighed 2-3 times/week
- All mice will be tested for grip strength weekly
- Mice will be scanned by DXA every 2weeks and by microCT every 4 weeks for the length of the experiment.
- 10 & 3 days before euthanasia all mice will be injected with calcein IP.
- Mice will be euthanized at 4 weeks (10 mice per group) and 8 weeks (10 mice per group) after surgery.
- All bones and organs will be harvested for histological and histomorphometrical analysis.

- Right EDL muscle will be used for ex vivo contractility assay.
- Left EDL muscle will be used for RyR1/Calstabin1 biochemistry.

Experiment 3

To test the effect of S107 and Zoledronic Acid (ZA), alone or in combination, on muscle specific force in sham operated \pm Flutamide male athymic mice $n=20/\text{group} \times 8 \text{ groups} = 160$ total

- Nine week old mice will be ordered.
- After arrival, mice will be acclimated for 2 days.
- Mice will be aged for 8 weeks, until adult (i.e. 17 weeks old).
- 49 days after arrival blood sample will be collected and mice will be weighed.
- 52 days after arrival baseline μCT .
- 53 days after arrival DXA and grip strength will be performed for all mice.
- 56 days after arrival orchiectomy (ORX)/ Sham surgery and insertion of mini-pumps will be performed consecutively on all mice and drug treatments will commence for all groups.
- All treatment will start 56 days after arrival

Group A: Sham surgery + vehicle (PBS) by s.c. injection.

Group B: Sham surgery + Flutamide (45mg/kg/day) by s.c. injection + vehicle (PBS) by s.c. injection.

Group C: Sham surgery + ZA (5 $\mu\text{g/kg}$ / 3 times/week) by s.c. injection + vehicle (PBS) by s.c. injection.

Group D: Sham surgery + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group E: Sham surgery + Flutamide (45mg/kg/day) by s.c. injection + ZA (5 $\mu\text{g/kg}$ / 3 times/week) by s.c. injection + vehicle (PBS) by s.c. injection.

Group F: Sham surgery + Flutamide (45mg/kg/day) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group G: Sham surgery + ZA (5 $\mu\text{g/kg}$ / 3 times/week) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

Group H: Sham surgery + Flutamide (45mg/kg/day) by s.c. injection + ZA (5 $\mu\text{g/kg}$ / 3 times/week) by s.c. injection + S107 (50mg/kg/d) s.c. osmotic minipump + vehicle (PBS) by s.c. injection.

- Mice will be weighed 2-3 times/week
- All mice will be tested for grip strength weekly
- Mice will be scanned by DXA every 2weeks and by microCT every 4 weeks for the length of the experiment.
- 10 & 3 days before euthanasia all mice will be injected with calcein IP.
- Mice will be euthanized at 4 weeks (10 mice per group) and 8 weeks (10 mice per group) after surgery.
- All bones and organs will be harvested for histological and histomorphometrical analysis.
- Right EDL muscle will be used for ex vivo contractility assay.
- Left EDL muscle will be used for RyR1/Calstabin1 biochemistry.

Animal protocol has been already approved by IACUC.

4. KEY RESEARCH ACCOMPLISHMENTS

Nothing to report.

5. CONCLUSION

The concluded experiments have evidenced loss of muscle mass and decreased bone mineral density in mice undergoing ORX. However, there is no significant decrease in muscle specific force in these mice compared to sham operated mice. Therefore, we will adopt a different approach in the next experiments, pairing ORX with an androgen receptor antagonist (Flutamide), mirroring clinical therapies adopted in patients with prostate cancer. We will also use two endpoints to measure muscle specific force, 4 weeks and 8 weeks, in order to be able to investigate the changes in muscle specific force when BMD reaches the lowest level and TGF β levels are the highest. If our hypothesis is verified, this could possibly indicate that early calcium leak through RyR1 and decreased muscle specific force could cause long-term consequences on muscle mass and overall quality of life in patients with prostate cancer undergoing ADT.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Nothing to report.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

Nothing to report.

9. OTHER ACHIEVEMENTS

Nothing to report.

10. REFERENCES

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11. APPENDICES

Nothing to report.